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TI The search for novel adjuvants for early life vaccinations: can "danger" motifs show us the way?.
AU Kovarik J; Siegrist C A
SO ARCHIVUM IMMUNOLOGIAE ET THERAPIAE EXPERIMENTALIS, (2001) 49 (3) 209-15.

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Vaccine 17 (1999) 1376–1383

Vaccine

Immunoadjuvant action of plasmid DNA in liposomes

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Abstract

Bacterial DNA and oligodeoxynucleotides containing immunostimulatory sequences with a CpG motif stimulated a Th1 type response in vivo. The adjuvant action of a non-coding plasmid DNA derived from pRc/CMV HBS (encoding the S region of hepatitis B surface antigen, HBsAg) in mice was investigated. The role of methylation on the adjuvanticity of the plasmid as well as the effect of vaccine formulation employed on the outcome of antigen-specific humoral and cellular responses were also studied. The results demonstrated that plasmid DNA acted as a Th1 promoting adjuvant when mixed as such or co-entrapped in liposomes with a very low dose of antigen. However, the adjuvant activity was lost when separate liposome entrapped formulations of both the antigen and the plasmid DNA were mixed, indicating a necessity for the antigen and the plasmid DNA to contact the same APC for optimal immune activation. A decreased adjuvanticity of plasmid DNA upon methylation with HpaII methyltransferase was also demonstrated. A mechanism that may help partially explain the reduction in adjuvanticity after modification of C residues is also discussed.

Keywords: Immunological adjuvants; DNA vaccine; Liposomes

1. Introduction

Genetic immunization with plasmid DNA encoding bacterial, viral, parasite and tumour antigens has been reported to trigger antigen-specific humoral and cell-mediated protective immunity [1–4]. This novel strategy of vaccination induces a Th1 type response characterized by IFN-γ production by $CD4^+$ T-cells and induction of $CD8^+$ T-cells [5]. The cell-mediated responses to the encoded antigen appears to be maximal when the plasmid backbone contains short immunostimulatory sequences (ISS) with unmethylated CpG motifs [6]. Bacterial DNA has a much higher frequency of unmethylated CpG dinucleotides than the vertebrate DNA due to CpG suppression and the methylation of 80% of the CpG in vertebrates [7]. In addition, an unmethylated CpG dinucleotide flanked by two 5′ purines (optimally a GpA) and two 3′ pyrimidines (optimally a TpC or TpT) are 20 times more common in bacterial than mammalian DNA [8] and were demonstrated to stimulate the innate immune system to produce an array of immuno-stimulatory cytokines [9]. Thus, these special ISS present in bacterial DNA appear to alert professional APCs to 'danger' and shift the class of immune response to that dominated by Th1 cells. The receptors that interact with both high molecular weight DNA and/or ISS containing oligodeoxynucleotides (ODN) appear to be immunoglobulin receptors on the surface of B-cells and the macrophage scavenger receptor on the surface of macrophages, leading to polyclonal B-cell activation and nuclear translocation of the transcription factor NFκB known to control the induction of TNF-α mRNA in macrophages [10, 11]. Owing to their ability to induce cytokines like IL-12, TNF-α and IFN-γ, bacterial DNA and ISS ODNs can be considered as a new class of immuno-adjuvants when a Th1 type response is desired.

Liposomes constitute another class of attractive immuno-adjuvants that can provide a vehicle or a carrier system into which antigens and co-adjuvants can be incorporated [12]. The co-adjuvant activity of IL-2

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and IL-15 [13, 14] in a variety of liposomal formulations incorporating a model antigen (tetanus toxoid) was determined previously. For these cytokines to act as a co-adjuvant, their presence together with the antigen in the same liposomes (co-entrapped, co-surface linked or adsorbed) was essential. The finding that none of the cytokines used could potentiate immune responses against the toxoid when entrapped separately from the antigen, suggested a need for the cytokine and the antigen to contact the same APCs simultaneously.

The main purpose of the present study was to assess whether a non-coding plasmid DNA derived from pRc/CMV HBS (encoding the S region of hepatitis B surface antigen, HBsAg) is capable of inducing effective HBsAg specific humoral and cellular immunity in its native or methylated forms, as such, or in the context of liposome entrapment.

The work involved digestion of pRc/CMV HBS plasmid with *Bam*HI and ligation of the largest fragment containing the ampicillin resistance (*AmpR*) gene, previously shown to contain two ISS (5′-AACGTT-3′), to obtain a non-coding plasmid DNA (pDNA). This HBS(-) plasmid was also methylated by HpaII methytransferase (MHpaII) generating MetpDNA. To assess the adjuvant action of pDNA or MetpDNA, mice were immunized intramuscularly with: (i) DNA mixed with HBsAg, (ii) DNA and HBsAg entrapped in different liposome populations but injected simultaneously and (iii) DNA and HBsAg co-entrapped in the same liposomes. Humoral and cellular immune responses measured against HBsAg were compared to those obtained with naked or liposome entrapped DNA vaccine (intact pRc/CMV HBS).

2. Materials and methods

2.1. Reagents

Recombinant HBsAg (subtype ayw) derived from *Hansenula polymorpha* was purchased from Rhein-Biotech (Germany). HpaII methyltransferase was from MBI Fermentas (Lithuania). Mouse IL-4 and IFN-γ ELISA kits were purchased from Amersham Life Sciences (UK). Peroxidase conjugated goat anti-mouse IgG_1, IgG_{2a} and IgG_{2b} were from Sera-Lab (UK).

2.2. DNA vaccine

Plasmid pRc/CMV HBS expressing sequences coding for the S (small) protein of hepatitis B virus (subtype ayw) [2] (Fig. 1) was a kind gift from Dr. Robert Whalen, Centre National de la Recherche Scientifique, Paris, France.

2.3. Preparation of the non-coding plasmid

pRc/CMV HBS plasmid was digested with *Bam*HI and the fragments generated were resolved on a 0.9% (w/v) agarose gel. The largest fragment containing the *AmpR* gene was then eluted from the gel, purified and self-ligated with T4 DNA ligase. Competent *E. coli* DH5α cells were transformed with the resulting plasmid and transformants were selected on ampicillin-containing agar plates. The DNA vaccine and the HBS(-) noncoding plasmid were propagated in *E. coli* DH5α and plasmids were isolated by the alkaline lysis method of Birnboim and Doly [15] followed by phenol–chloroform extraction (twice) and ethanol precipi-

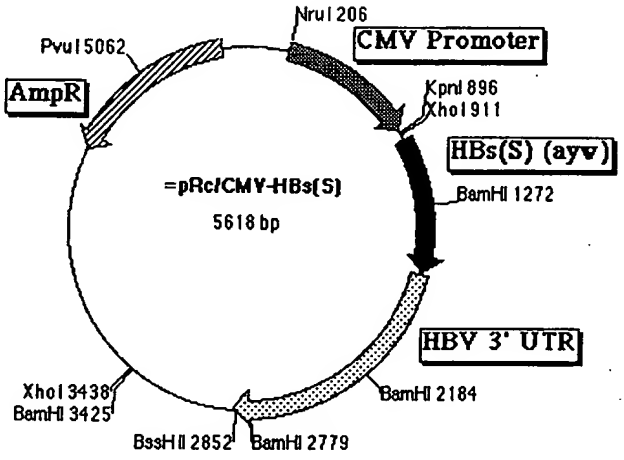

Fig. 1. Map of HBsAg-expressing plasmid pRc/CMV-HBs(s).

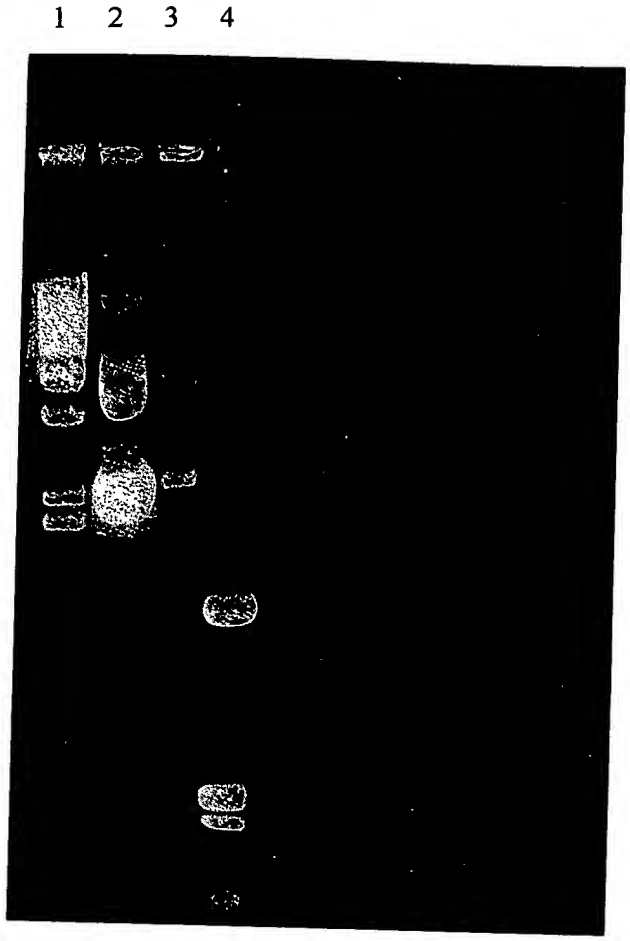

Fig. 2. Verification of methylation of pDNA after treatment of the methylated plasmid by HpaII. The methylated and unmethylated pDNAs were treated with HpaII at 37°C for 1 h and cleavage fragments were analysed on a 1% agarose gel. Lane 1: *Hind*III markers; lane 2: mock treated pDNA; lane 3: MetpDNA treated with HpaII; lane 4: pDNA treated with HpaII.

tation. In some experiments, the non-coding plasmid was methylated with MHpaII as recommended by the manufacturer. Methylation was verified by treating the methylated plasmid with HpaII (cleaves from C^{l}CGG site if the internal C is not methylated) (Fig. 2).

2.4. Entrapment of plasmid DNAs and HBsAg into liposomes

Multilamellar liposomes with entrapped pDNAs and/or HBsAg were prepared according to the method of Kirby and Gregoriadis [16]. In brief, small unilamellar vesicles (SUV) composed of equimolar phosphatidylcholine (PC) and cholesterol (total lipid content: 32 μmol) were mixed with 250 μg DNA alone or together with 2.5 μg antigen (in the case of antigen entrapment alone, SUVs were mixed with 10 μg HBsAg, Table 1). The mixtures were freeze-dried overnight and then rehydrated under controlled conditions to generate dehydration–rehydration vesicles (DRV liposomes). These were then washed by centrifugation (27,300 × *g*) and resuspended in 0.05 M PBS, pH 7.4. Estimation of percent solute entrapment was based on the quantitation of antigen and DNA in the unentrapped fraction by the bicinchoninic acid protein assay [17] and absorbance at 260 nm, respectively.

2.5. Immunization experiments

Outbred mice (male, 20–25 g body weight) randomly divided in groups of 4, were injected i.m. twice (on days zero and 28) with naked (10 or 50 μg/mouse) or liposome-entrapped DNA vaccine (10 μg) or with non-coding plasmids (10 μg) in their methylated and unmethylated forms in the presence of 0.1 μg HBsAg as such or in combination with liposomes.

Blood samples were collected from the tail vein on days 28 (primary response) and 39 (secondary response) and sera were kept at −20°C until they were tested by ELISA for anti-HBsAg IgG_1, IgG_{2a} and IgG_{2b}. Animals were killed (on day 40) and spleens

Table 1
Incorporation of HBsAg and DNA into DRV liposomes

Preparations	HBsAg used[a] (μg)	Entrapped HBsAg (%)	DNA used[a] (μg)	Entrapped DNA (%)
E DNA vaccine[b]	–	–	250	49.9
SE Ag + pDNA[c]	10.0	48.7	350	65.5
CoE Ag + pDNA[d]	2.5	45.5	250	48.2
SE Ag + MetpDNA[e]	10.0	48.7	350	63.8
CoE Ag + MetpDNA[f]	2.5	45.0	250	49.1

[a] Amounts of HBsAg and DNA used were adjusted to give, a final solution containing 0.1 μg antigen (except for the DNA vaccine) and 10 μg DNA 0.1 ml^{-1} injected volume.
[b] DNA vaccine entrapped in DRV liposomes (E DNA vaccine).
[c] pDNA and HBsAg entrapped in separate liposome populations (mixed before injection) (SE Ag + pDNA).
[d] pDNA and HBsAg entrapped in the same liposomes (CoE Ag + pDNA).
[e] MetpDNA and HBsAg entrapped in separate liposome populations (mixed before injection).
[f] MetpDNA and HBsAg entrapped in the same liposomes.

were removed for the determination of endogenous levels of INF-γ and IL-4.

2.6. ELISA

ELISA plates (96-well flat bottom, Nunc, Polysorb) were coated with HBsAg (60 μl/well, 2 μg/ml) dissolved in 0.05 M carbonate–bicarbonate buffer pH 9.6 and incubated at RT for 1 h. The plates were washed three times in PBS–Tween 20 buffer (0.05% v/v, wash buffer) and blocked with 1% (w/v) BSA in wash buffer (60 μl/well) for 1 h. Two-fold serially diluted mouse sera (60 μl/well) were added, and incubated 2 h at RT. Following washing, peroxidase-conjugated goat anti-mouse IgG_1, IgG_{2a} or IgG_{2b} was added (50 μl/well, 1 in 2000 diluted in wash buffer containing 1% (w/v) BSA and 5% (v/v) FCS). At the end of a 3 h incubation and subsequent washing, 200 μl/well of the substrate *o*-phenylenediamine (40 mg/100 ml freshly prepared citrate-phosphate buffer (pH 5.0) containing 40 μl 30% H_2O_2) was added and the reaction was stopped after 30 min with 1.5 M H_2SO_4 (25 μl/well). Colour development was determined spectrophotometrically at 490 nm. Results (means ± S.D.) were expressed as $\log_{10}$ of the reciprocal number of the final detectable dilution, which gave an $A_{490\ nm}$ of ≥0.2 units.

2.7. Cytokine extractions and cytokine assays

Endogenous levels of IL-4 and IFN-γ in mouse spleen homogenates were determined with two separate ELISA kits for these cytokines as described by the manufacturer. Spleen homogenates were prepared by the method of Nakane et al. [18] as previously modified by de Souza and Playfair [19]. In short, spleens from control and immunized mice were weighed and homogenized in ice-cold PBS containing 1% CHAPS (10% (w/v) homogenates). Homogenates were incubated on ice for 1 h and centrifuged at 2000 × *g* for 20 min. The clear supernatant fluids obtained were used in cytokine assays by ELISA.

2.8. Data analysis

ELISA values were statistically analyzed using the Student's *t*-test.

3. Results

3.1. DNA and HBsAg content of liposomes

Values of HBsAg and DNA entrapment for each of the liposome formulations prepared are shown in Table 1. Entrapment values for HBsAg were considerably high (48.7%) even when the protein was co-entrapped with DNA (45.0–45.5%). Since HBsAg particles derived from *Hansenula polymorpha* are 60% protein and 40% lipid by weight with PC being the major phospholipid [20], some of the antigen would be expected to be incorporated into the lipid bilayers of DRV, thus contributing to the high protein entrapment values observed. DNA entrapment values were also high (49.9% for DNA vaccine) and similar to those obtained by Gregoriadis et al. [21] employing the same method for entrapment of the same DNA vaccine used in this study.

However, entrapment of the non-coding plasmid (methylated or unmethylated) was much higher (63.8–65.5%) probably due to its smaller size (non-coding plasmid is 3465 bp as compared to the 5618 bp DNA vaccine). In the case of coentrapment, these values were lowered (48.2–49.1%) due to the presence of HBsAg, due to its particulate nature, which limits the available accommodating space for the DNA in the aqueous phase of the liposomes.

3.2. The effect of pDNA and MetpDNA on serum antibody responses against HBsAg

The adjuvant activity of unmethylated or methylated non-coding pDNAs was investigated in experiments where mice were immunized with 0.1 μg HBsAg in the presence of the adjuvant DNA (10 μg) alone or in 2 different liposomal formulations. The latter included separately entrapped antigen and DNA (mixed before injection) and HBsAg and DNA co-entrapped in the same liposomes. This antigen dose (0.1 μg), is very low and did not induce detectable antibody when injected alone as determined in preliminary experiments (data not shown). We reasoned that use of a suboptimal antigen dose was essential in order to observe the effect of the adjuvant component itself. Responses obtained with the above formulations were compared with those of naked (10 or 50 μg) or liposome entrapped (10 μg) DNA vaccine.

Analysis of primary responses (Fig. 3a) revealed that pDNA stimulated increased levels of all IgG subclasses tested in comparison to naked DNA vaccine (10 μg) when mixed with the antigen or co-entrapped together ($P < 0.05$–0.001) but not when entrapped in separate vesicles. Methylation abolished the adjuvanticity of the pDNA in terms of IgG_{2a} subclass production. Co-entrapped HBsAg and Met-pDNA was still effective in boosting IgG_1 titres ($P < 0.05$–0.001) when compared to all DNA vaccine preparations employed here.

The adjuvant action of pDNA was much more evident when the secondary responses were analyzed (Fig. 3b). There was a modest but significant increase in IgG_1 titres when antigen–pDNA mixture (9-fold) and co-entrapped (3-fold) formulations were used in

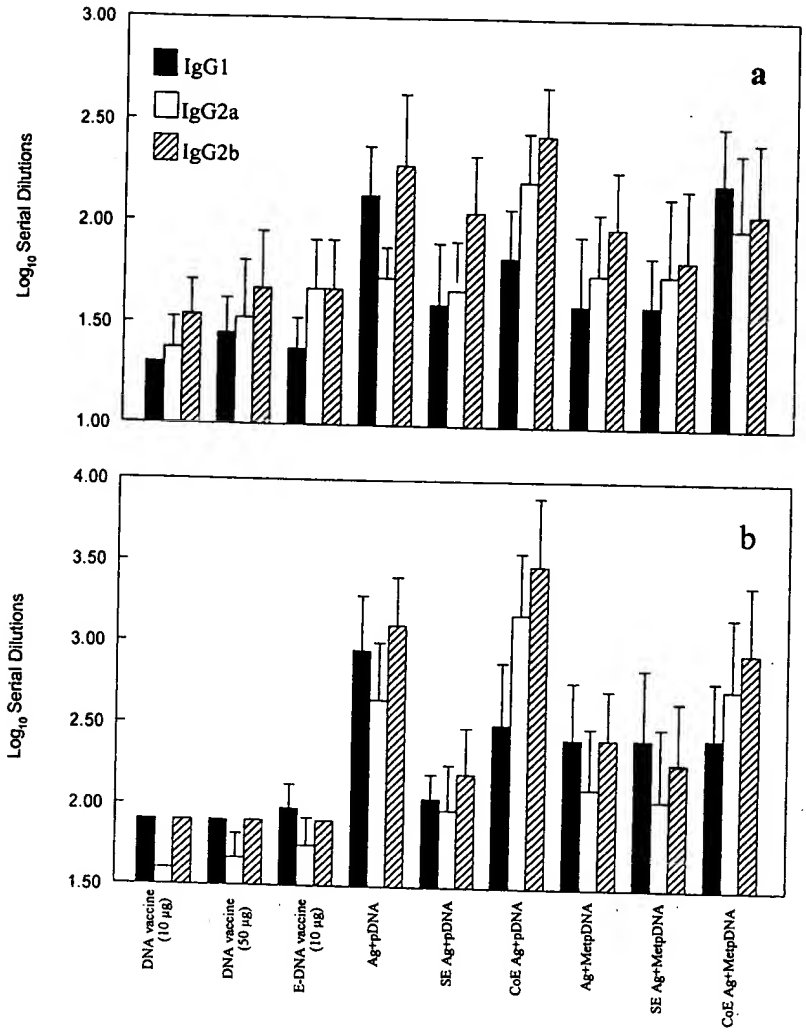

Fig. 3. Comparison of primary (panel a) and the secondary (panel b) immune responses in mice injected with DNA vaccine or HBsAg in the presence of pDNA (unmethylated or methylated) as adjuvant. Mice (four per group) were injected i.m. on days 0 and 29 with naked (10 or 50 μg/mouse)or liposome entrapped (10 μg/mouse) DNA vaccine or with HBsAg (0.1 μg) mixed, separately entrapped (SE) or co-entrapped (CoE) with pDNA or MetpDNA (10 μg). Mice were bled on days 28 and 39 and anti-HBsAg antibody responses were analysed by ELISA. Results are expressed as the mean ± S.D. of individual $\log_{10}$ titres.

comparison to all DNA vaccine formulations. However, these same formulations boosted the titres of IgG_{2a} 8- and 27-fold and the titres of IgG_{2b} 16- and 37-fold, respectively, when compared to DNA vaccines. Owing to its ability to raise the titres of the IgG2 subclasses, co-entrapment appears to yield a stronger Th1 response than the Ag–pDNA mixture. The separately entrapped formulation, on the other hand, was completely ineffective. These results, where an improvement in humoral responses could be achieved with a mixture of antigen–pDNA or with a co-entrapped formulation but not with a separately entrapped preparation, might as well imply that the HBsAg interacts with the pDNA upon mixing and thus contact the same APC simultaneously.

Methylation of the internal C residue of the sequence 5′-CCGG-3′ reduced the adjuvant action of the pDNA. The three MetpDNA containing formulations proved to be ineffective in raising the titres of IgG_1 (results were statistically non-significant when compared to DNA vaccinated groups). However, the co-entrapped antigen and MetpDNA could still improve the IgG_{2a} and IgG_{2b} titres (9-and 11-fold, respectively). The antigen–MetpDNA mixture boosted only the IgG_{2b} production modestly (a 3-fold increase in comparison to the DNA vaccine preparations). These results, where a reduction of adjuvanticity was observed upon methylation of a non-immunostimulatory sequence (the pDNA contains at least 9 such sequences), implies that the behaviour of CpG sequences flanked by bases previously reported to be non-immunogenic using ODNs in vitro [7], might be quite different using a plasmid DNA in vivo and might point to the importance of overall DNA conformation.

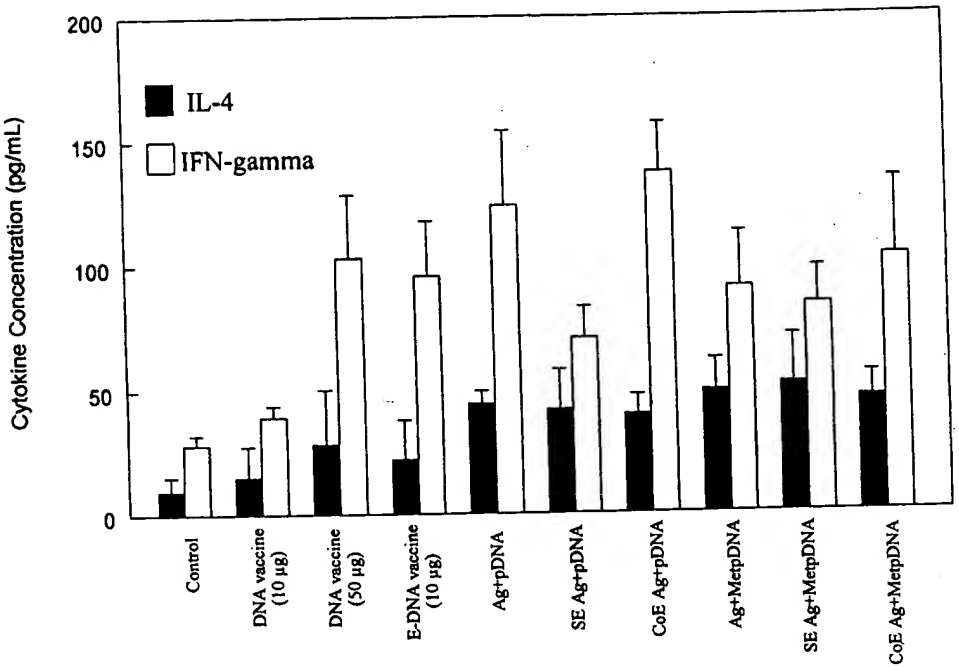

Fig. 4. Endogenous cytokine levels in mice immunized with DNA vaccine or HBsAg in the presence of pDNA (unmethylated or methylated) as adjuvant. Mice were immunized as in Fig. 3 and spleens from naive (control) and immunized groups were harvested at day 40. IL-4 and IFN-γ levels were measured in homogenates. Results are expressed as the mean ± S.D. of a group of 4 mice.

3.3. Cytokine profiles

IFN-γ production is a well-known property of Th1 cells after antigenic stimulation [22]. Use of pDNA either mixed or co-entrapped with HBsAg produced the highest IFN-γ levels (Fig. 4) indicative of a Th1 dominated response. Methylation slightly reduced the levels of this cytokine but not of IL-4. All pDNA containing formulations (either methylated or unmethylated) yielded similar IL-4 levels which were consistently higher than those induced by DNA vaccination (Fig. 4). These results imply that the adjuvant DNA can induce a strong Th1 response and also stimulates Th2 cells modestly.

4. Discussion

The realization that certain ISS containing an unmethylated CpG motif were capable of inducing Th1 promoting cytokines and activate B-cells, macrophages and NK cells [7,9–11,23,24] prompted several research laboratories to assess the adjuvant action of DNA in vaccine formulations [8,25–28]. However, the exact mechanism of how bacterial DNA or ISS containing ODNs alert the immune system to a Th1 type response is still obscure. A few studies suggest that unmethylated CpG motifs are necessary but not sufficient for activation of immune cells [11,29]. Sparwasser et al. [11] reported that an ISS ODN bearing an unmethylated CpG motif activated murine macrophages to produce TNF-α but a very similar sequence derived from the eukaryotic cAMPC response element sequence (which also contains an unmethylated CpG) motif had no effect at all. In another study, hypomethylated DNA from two mammalian cell lines were ineffective in stimulating murine B-cells [29] despite containing the necessary CpG motif. There are also contradicting reports on the adjuvant action of bacterial DNA. For instance, *Escherichia coli* DNA was reported to enhance IgG_{2a} antibody production to β-galactosidase [25] but had no detectable adjuvant effect where a tumour antigen was used in immunization [26]. Taken together, all the available information imply that the adjuvant action of bacterial DNA cannot be solely attributed to a lack of CpG methylation and further work is required to clarify its immunostimulatory role.

Here we have assessed the adjuvant action of an unmethylated and methylated pDNA as such or in the presence of liposomes. The reason for liposome usage in immunization studies were 3-fold: first, these vesicles were proven to further potentiate the adjuvant activity of other immuno-modulators [12–14]. Second, using different formulations, one can deduce whether there is a requirement for the antigen and the adjuvant to contact the same APC to induce an optimal immune activation and finally, encapsulation within liposomes can

protect the DNA from nuclease attack in vivo [21]. The study also focused on the effect of methylation on the adjuvanticity of pDNA. To the best of our knowledge, all the available data published until now (about the effect of methylation on the adjuvanticity of DNA) involved methylation of DNA with SssI methylase which modifies all the C residues in the recognition sequence 5′-CG-3′. However, we have attempted to determine the effect of methylation of a subset of CpG motifs (5′-CCGG-3′) on the adjuvant action here. Despite lack of evidence for an immuno-stimulatory role for this sequence, a CCG and a CGG trimer occurs with slightly higher frequency in *E. coli* DNA than eukaryotic DNA [29].

Our results demonstrate that pDNA–HBsAg mixture and especially their co-entrapment in the same liposomes can augment both humoral and cellular immune responses to the antigen significantly when compared to DNA vaccination. The finding that pDNA was effective only when mixed or co-entrapped with the antigen but not when the two were separately entrapped, strongly suggests a physical association of HBsAg with the DNA. Despite lack of data on DNA–HBsAg interaction in the literature, there is other evidence implying that such an event is highly plausible: for example, an electrostatic interaction between HBsAg and negatively-charged phospholipids was reported previously [30]. More interestingly, the solid-phase ethylene/maleic anhydride polyelectrolytes has been shown to adsorb and remove a large proportion of HBsAg from human plasma or contaminated plasma cryoprecipitates [31,32] indicating that this viral protein has a tendency to interact with negatively-charged surfaces. DNA in itself can be considered as a polyelectrolyte with a very high negative charge density and therefore, an electrostatic interaction between HBsAg and pDNA is therefore plausible. In the case of other antigens, such an interaction would be unexpected and co-entrapment of the DNA adjuvant and the antigen within liposomes might be an attractive approach to improve specific immune responses.

Our data also demonstrate a decreased adjuvanticity of pDNA upon methylation with MHpaII. Despite lack of evidence for an immuno-stimulatory role for 5′-CCGG-3′, this sequence appears to be important for mammalian DNA: although more than 95% of all animal DNA methylation occurs at the sequence CG, several reports indicated C modification especially at MspI (CCGG) sites located at the 5′-flanking region of the human γ-globulin genes [33]. Also of interest is the observation that in vitro enzymatic methylation of the CpG dinucleotide by MHpaII inhibits expression of viral genes after introduction of the methylated DNA into cells [34]. Thus, methylation of CCGG residues might as well curtail the 'danger signal' delivered by the pDNA and reduce the adjuvanticity of this molecule. More importantly, we also suggest that C methylation might alter the binding capacity of DNA to cytoplasmic or cell-surface expressed signal transduction molecules present in or on certain cells of the immune system, thereby affecting transcription of various cytokine genes. C methylation is known to facilitate the B (right-handed) to Z transition (left-handed) in supercoiled recombinant plasmids [35], enhance sequence-directed DNA curvature [36] and therefore constitute a concerted mechanism for the modulation or fine tuning of DNA topology and DNA–protein interactions. Since the components of a cell that interact with DNA are thought to be proteins, methylation of certain bases might alter the DNA configuration to a state less interactive (i.e. less recognizable) and therefore less immunogenic. Further work using different methylases (other than SssI) to modify certain base sequences in the structure of pDNAs might improve our understanding of the immunostimulatory properties of procaryotic DNA.

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